Molecular techniques used for the detection of babesia spp. in cattle: systematic review

Técnicas moleculares empleadas para la detección de babesia spp. en bovinos: revisión sistemática

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Abstract

Bovine babesiosis is one of the most important parasitic diseases in the world, it is caused by protozoa of Babesia genus and generates anemia, anorexia, weight loss, jaundice and hemoglobinuria generating loss in production and even the death of the animal. Serological tests are relatively inexpensive and efficient to detect the parasite, but the results depend on factors

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inherent to the animal and the state of infection. Molecular markers are more precise tools to detect the parasite. The aim of the current systematic review was to review original studies, published between 2010 and 2018, in which molecular techniques were implemented to detect the parasite. The research was carried out using PubMed, LILACS, SciELO and Science Direct databases. 196 records were identified according to the research criteria and only 47 were analyzed. The main molecular techniques reported include Polymerase chain reaction (PCR), nested PCR (nPCR) and semi nested PCR, Real time PCR (qPCR), Reverse Line Hybridization (RLB), Cloning, Sequencing and Amplification isothermal nucleic acid (LAMP). The main molecular targets include the AMA-1 region, the 18s rRNA genes, the RAP-1 marker, the ITS region, SBP-2 and 4 proteins, VESA-1 and MSA. From the information obtained, the high impact of the disease, the efforts of different countries to study it and to investigate its causal agent is evident. The need to increase research in aspects in which molecular techniques are useful, such as the knowledge of the parasite genome and its dynamics with the host, in order to advance in the development of control and/or eradicationstrategies is also fundamental.

Keywords: Babesiosis, Diagnosis, Hemoparasites, Molecular marker

Resumen

La babesiosis bovina es una de las enfermedades parasitarias más importantes en el mundo, es ocasionada por protozoos del género Babesia y genera signos como fiebre, anemia, anorexia, pérdida de peso, ictericia y hemoglobinuria, con las consecuentes pérdidas en producción, e incluso la muerte del animal. Para la detección del parásito, se usan pruebas serológicas, relativamente económicas y eficientes, cuyos resultados dependen de factores inherentes al animal y al estado de la infección. Las técnicas moleculares constituyen herramientas más precisas para la

detección del material genético del parásito. Con el objetivo de evaluar los estudios en los cuales se implementaron técnicas moleculares para la detección de parásitos del género *Babesia*, se realizó una revisión sistemática de investigaciones realizadas entre los años 2010 y 2018, en las bases de datos PubMed, LILACS, SciELO y ScienceDirect. De 196 registros identificados solo 47 cumplieron con todos los criterios. Las principales técnicas moleculares empleadas comprenden la Reacción en cadena de la polimerasa de punto final (PCR), PCR anidada (nPCR) y semi anidada, PCR en tiempo real (qPCR), Hibridación en línea inversa (RLB), Clonación, Secuenciación y Amplificación isotérmica de ácidos nucleicos (LAMP). Los principales blancos moleculares incluyen la región AMA-1, los genes 18s rRNA, el marcador RAP-1, la región ITS, proteínas SBP-2 y 4, VESA-1 y MSA. Se evidencia el gran impacto de la enfermedad, los esfuerzos en diferentes países por estudiarla y conocer su agente causal, asi como la necesidad de ampliar la investigación en aspectos en los que las técnicas moleculares son de gran utilidad, como el conocimiento del genoma del parásito y su dinámica con el hospedador, a fin de avanzar en el desarrollo de estrategias de control y/o erradicación.

Palabras clave: Babesiosis, Hemoparasitos, Diagnóstico, Marcador molecular

1. Introduction

Babesia spp. is one of the most problematic hemoparasites affecting tropical and subtropical livestock. It causes Bovine Babesiosis, a disease that together with anaplasmosis forms the Bovine Parasitic Sadness Complex (Blanco *et al*., 2016). The parasite infects red blood cells causing clinical signs such as fever, anorexia, weight loss, decreased milk production, tachypnea, jaundice, hemoglobinuria and can end in the death of the animal (Zhou *et al*., 2016; Bock *et al*., 2004; Kocan *et al*., 2010).

Disease control in many parts of the world is limited to chemotherapeutic treatment and control of the tick population with acaricide agents, but there are no control programs based on herd immunity studies, comprehensive control of the tick and the diseases it transmits, or commercially available babesiosis vaccines (Mosqueda *et al.,* 2012). Early diagnosis is the most effective tool for disease control, however, serological tests that have been used in many epidemiological studies to assess prevalence, despite being relatively inexpensive and efficient, fail to detect early infection (pre-seroconversion) (Ramos *et al.,* 2010) and their results depend on factors inherent to the animal and the infection status. In the last decade, many molecular markers have been developed, these are precise tools with an increasing specificity and sensitivity, that allow the detection and identification of parasites that were previously difficult to diagnose with conventional techniques (Tavares *et al*., 2011). In this systematic review, the molecular methods and the regions of the genome of the microorganism used for the detection of *Babesia* sp in cattle, addressed in researches conducted during the years of 2010-2018 were analyzed. This information will be key to facilitate the conduction of studies focused on the search of more efficient control and possible eradication measures.

2. Materials and Methods

A systematic search was conducted for studies that met the following inclusion criteria: original studies, quantitative, completed, in Spanish, English and Portuguese and published between 2010 and 2018, in which the detection of hemoparasites belonging to the genus *Babesia* was carried out. Search criteria: The search was carried out in the PubMed, LILACS, SciELO and ScienceDirect databases, using the following terms: (tw:(Babesia)) and (tw:(Cattle)) and (tw:(POLYMERASE CHAIN REACTION)) or (tw:(REAL TIME POLYMERASE CHAIN REACTION)) or (tw:(REVERSE TRANSCRIPTASE POLYMERASE CHAIN

REACTION)) or (tw:(WESTERN BLOTTING)) o r (tw:(MOLECULAR DIAGNOSTIC TECHNIQUES)) or(tw:(MICROARRAY ANALYSIS)). For the selection of the studies we worked using the PRISMA flowchart (Moher *et al*., 2009). The collection and synthesis of the results was done through Excel tabulation.

3. Results and Discussion

Out of 196 records found, 167 articleswhose abstracts were relevant to the topic were selected, of which only 47 m*et al*l the criteria.

Distribution of the studies found

Of the selected studies, 25 were carried out between 2015 and 2017, being 2017 the most productive year with 10 published studies. According to the geographical distribution, 17 studies published in ten countries of the Asian continent stood out, mainly in the Philippines and Sri Lanka; 14 researches were reportedin eight African countries, 12 in the American continent, published in countries like Colombia, Mexico, Argentina, Costa Rica and Brazil (country with the biggest production of the region), and finally three European countries with four studies.

Characteristics of the evaluated population

Only 14 studies specified the type of cattle evaluated (seven on crossbreed cattle, five on native or creole breeds and the remaining studies in Senepol, Braford, Angus and Holstein cattle). According to the productive stage of the animals, 22 studies did not specify this criteria, 19 sampled cattle of different ages, four limited the sample to calves, one limited to steer and only one study was based solely on adult cattle. The size of the used sample in approximately half of the studies (53.19%) was composed of

around 100 and 400 individuals, 19,15% of approximately 400 and 500, 14,9% between 50 and 100 and 6,38%, of each case used more than 700 or less than 50 individuals.

Molecular techniques used

The molecular techniques reported in the analyzed studies include End Point Polymerase Chain Reaction (PCR), Nested (nPCR) and Semi Nested PCR, Real Time PCR (qPCR), Reverse Line Hybridization (RLB), Cloning, Sequencing and Nucleic Acid Isothermal Amplification (LAMP).

According to Bolivar (2013), PCR is a diagnostic technique with a high degree of specificity and sensitivity, lacking the diagnostic interference that is usually implied when other methods are applied, including conventional parasitological and/or serological methods. A widely used variant of PCR is real-time PCR (qPCR), which offers the possibility of quantifying the amount of DNA or RNA present in the sample (Guevara *et al*, 2011), however, Giglioti *et al* (2017) evaluated the repeatability and correlation between the number of copies of a fragment of the genes that code for bovine cytochrome B (NC mt-cyB) and the antibody titre as a possible indicator of susceptibility/resistance of Angus cattle to the parasite, finding low values of repeatability and correlation between both indicators, so they infer that such tests cannot be used to determine resistant or susceptible phenotype to the parasite.

Nested PCR (nPCR) has two rounds of amplification where the product of the first reaction serves as a template for the second one, this way the parasitic genome can be routinely detected and the reproducibility and sensitivity depend on the amount and nature of the initial DNA mold (Snounou y Singh., 2002). Bath *et al.,* 2014 evaluated the effectiveness of PCR and nPCR in detecting *B bigemina* in a sample of 204 cattles, finding that the positive value of 7.35% found by primary PCR, increased to 30.39% with

nPCR, assuming that the amplification had already been achieved with primary PCR, although it could only be visualized in all samples by nPCR.

Hosary (2017) in a test carried out with 75 bovines from different localities of Asiut Governorate (Egypt), found a sensibility of 100% for the detection of *B bovis* through nPCR in the gene BV5650, compared to 65% obtained by PCR in the same gene. The lowest sensibility (30%) was obtained using blood smear and Giemsa staining, that is why for detection of bovines under field conditions and for epidemiological studies they recommend the first two methods.

Other studies have compared the effectiveness of molecular versus serological testing. Cao *et al* (2012), found a lower prevalence of *B bovis* and *B bigemina* using nPCR, possibly because the DNA of these parasites might have not been detected in cattle with chronic infections due to low parasitaemia.The persistence of antibodies in circulation even after the elimination of the parasites can explain such discrepancy, soin subclinically infected animals or animals with early infections nPCR can be applied for its detection, without the influence of the synchronization between the presence of the parasite and the antibody response, as well as the state of the infection (Cao *et al*., 2012).

A technique mentioned in the studies analyzed is Reverse Line Hybridization (RLB), which involves covalent bonding of specific capture oligonucleotide probes to a membrane, followed by hybridization and detection of PCR products using streptavidinlabeled peroxidase and a chemiluminescent substrate. After use, the membrane can be dismantled and reused at least 20 times (Kong y Gilbert, 2007). Its main advantage is that with some variants, it allows the simultaneous identification of different genus of bovine hemoparasites in a large number of samples, possibly making it an essential tool for epidemiological studies (Paolettaa *et al*., 2018).

Other tools such as the analysis of microarrays are useful for evaluating the gene expression involved in virulence and its possible post genomic modifications. Pedroni *et al* (2013) evaluated the profile of the virulent and attenuated *B bovis* transcriptome, finding a differential expression of 61 genes (41% and 59% expressed in the virulent and attenuated strains, respectively), highlighting regions such as SmORFs (genes that encode for small open reading frames for proteins), hypothetical genes (sequences that contain an open reading frame and that have been assigned a locus with its corresponding access number in the gene bank, without having been proven, experimentally or functionally, to actually code) (Pérez *et al.*, 2012) and $VESA_1$ (erythrocyte surface antigen variants), highlighting the role of the latter, involved in the cytoadherence of infected cells to endothelial cells and whose differential expression in the attenuated strain suggests a gene-specific activation, in addition to the activation of sbp-2 genes, which may play a role in the attenuated phenotype. Procedures such as fragment cloning are very useful to obtain the recombinant expression, in bacterial systems, of genes that encode membrane and organelle proteins involved in target cell invasion processes (Cruz *et al*., 2016), in the search for efficient vaccines.

According to Foxman *et al* (2005), there is no single molecular test that can fully satisfy the requirements that need to be known for all epidemiological and diagnostic applications, such as sensitivity, specificity, discriminatory power, cost, response time, reproducibility, ease of development and interpretation. However, the sensitivity of PCRbased methods can facilitate the analysis of vaccines and their ability to induce or prevent carrier status. In addition, they can be used to test the efficacy of antiparasitic drugs and in transmission and epidemiology studies (Adham *et al*., 2009).

Addressed genome regions

The studies that analyze the presence of *B bovis*, report the use of 9 different molecular targets, for the detection of *B bigemina* 6 and only one study detected *B ovata* by amplifying the AMA-1 region. The 18s rRNA genes are the most widely chosen for detecting the genus, as well as for searching for *B bigemin*, and to a lesser extent for *B bovis*.

The marker RAP-1 was frequently used for the detection of *B Bovis*and *B bigemina*. For the detection of this last one, besides the other two regions, the region AMA-1 and in less extent the region ITS are also used, a putative gene forBabesipsine, a non-described aspartic protease present in *B bovis*and*B bigemina* (Florin *et al.*, 2002), and a hypothetic protein on which there is no precise information. Regarding*B bovis*, sbp-4 and in less extent MtCyB, VESA-1, MSA 2C markers are relevant, and similar to*B bigemina*, Babesipsine and ITS markers are relevant in lower proportion.

18sRNA

The 18S small subunit ribosomal RNA (rRNA 18S) genes, for being highly conserved, have been used for evolutionary and taxonomic studies (Aktas *et al.,* 2007; Luo *et al*., 2005). Its abundance is 50% higher than the chromosomal DNA in the cell and its sequence has been proposed as a possible target for developing diagnostic tests for babesiosis. The information coming from this region is recommended for the molecular analysis and the reconstruction of the evolutionary history of the organisms, because by having a slow evolutionary rhythm, it allows the recognition of changes in place and time (Ríos y Ríos, 2011).

Using this region, Buling *et al* (2007) compared *B bigemina* sequences from different regions, finding a greater similitude between samples coming from America (Argentina, Mexico [obtained in the GenBank]) or Europe (Spain, Portugal) than

samples from other continents. Adham *et al.,* (2009) compared Egyptian strains of *B bovis* with Mexican strains, finding a high grade of similitude, so they recommend the PCR based method because it is highly sensible and widely applicable to parasite strains from different geographic regions.

The partial sequencing of the V4 region of the 18s ARNr gene in different species has demonstrated a significantly higher sensibility and specificity compared to microscopy, promising to be a very powerful tool for detecting lower rates of parasitemia and for discriminating species and coinfections (M´ghirbi *et al*., 2008).

Spherical body protein (SBP) gene

It is a compliment of different proteins (SBP1 and SBP4), secreted by the spherical body, an organelle attached to the membrane, located in the apical complex and exclusive of *Babesia* and *Theileria* spp. (Guo *et al*., 2018). SBP -2 and SBP -4 are part of the genes related to parasite infection and to immunogenicity (Simas *et al*., 2020). Once characterized in *B bovis,* it is assumed that this gene can contribute to intracellular survival, growth and development of this parasite, as well as the generation of erythrocyte membrane alterations (AbouLaila *et al*., 2010; Lobo *et al*., 2012). The SBP-4 protein is secreted by the parasite during its intra-erythrocyticphase (Cruz *et al*., 2016) and is distributed in the cytoplasm of the erythrocyte, this suggests that its importance lies in this part of the life cycle (Alaa *et al.,* 2011). Given that by bioinformatics in *B bigemina* a homologous protein to SBP-4has been found, it is expected that the production of this recombinant protein will allow the evaluation of a potential diagnosis and vaccine (Cruz *et al*., 2016).

Apical membrane antigen (AMA-1)

The apical membrane antigen is a protein apically located in many apicomplex organisms and its role lies in the invasion process of the host's erythrocytes (Torina *et al*., 2010). It has been found that AMA-1 genes are highly conserved, at least those isolated from *B bigemina* found in Argentina, Italy and Australia (Sivakumar *et al.,* 2012). Barreda *et al* (2019) studied peptides that contained B cell epitopes exposed and conserved from the AMA-1 extracellular region of *B bovis*, finding the presence of antibodies in bovine serum from endemic areas that attached to the identified peptides, with which they concluded that the epitopes are involved in the immune response under natural conditions, however, a greater characterization of the humoral immune response provoked by these peptides is needed.

Merozoite surface antigens (MSA

Multigenic family present in *B bovis,* formed by five members located in two different genomic loci: MSA-1 y MSA-2, that codify for a group of proteins that have been studied for the development of vaccines due to their antigenic properties (Mosqueda *et al.,* 2002), because they codify for 42 y 44 kDa glycoproteins, respectively, exposed in the surface of the merozoite and that are involved in the erythrocyte invasion (Florin *et al.,* 2002; Wilkowski *et al*., 2003; Genis *et al*., 2008). Both proteins come from sole copy genes with a hypervariable region associated to related immunogenicity and antibody neutralization characteristics.This indicates that this region is under great selective pressure and could be considered an excelent pathogenicity attenuation indicator (Simas *et al.*,2020).

For the isolation, cloning, sequencing and diagnostic analysis of the parasite, the MSA-2 gene is recommended, because according to Borgonio *et al*., (2008), previous studies on MSA-1 have evidenced allelic variation of antigens isolated from *B bovis* of similar endemic regions, and from isolates from different

geographical regions of the world. However, studies on MSA-2c, have demonstrated that this antigen is widely conserved in isolates from different geographical regions. This gene is involved in the survival of the parasite and its product is candidate for inclusion in a possible vaccine because it contains highly immunogenic and conserved epitopes that cause neutralization of sensitive antibodies in cattle (Wilkowski *et al*., 2003; Mosqueda *et al*,2012).

Variants of the erythrocyte surface antigen (ESA)

B bovis stablishes a chronic infection in cattle, in part through a rapid variation of the VESA1 polymorphic and heterodimeric protein. Its components, which are synthesized by the merozoite and are transported to the surface of the infected erythrocytes, are involved in the cytoadhesive function of the parasite. (Allred *et al*., 2000; O'Connor *et al*., 1997). Its rapid antigenic variation can be related to the evasion of the immune response and the cytoadhesion of the infected erythrocytes, this causes the sequestration of the infected erythrocytes in the brain capillaries and can lead to a much severe form of the disease (Pérez de la Rosa *et al*., 2012).

The complex is formed by a128 kDa (VESA1α) protein and a 113 kDa (VESA1β) protein. The first one is located over the parasitized erythrocyte's extracellular phase of the plasmatic membrane, at the level of the protuberances called "knobs (Figueroa y Alvarez., 2003). Babesia genomes are widely conserved in other aspects, but the evolution of VESA1 genes presents a constant change, which may reflect the challenges of host-parasite interactions that occurred over millions of years (Jackson *et al*., 2014).

Internal rRNA transcribed spacers (ITS)

Formed by the internal transcribed spacer 1 (ITS1), the rRNA gene 5.8S and the internal transcribed spacer 2 (ITS2), which because they are subject to higher evolutionary rates leading to greater variability in both nucleotide sequences and length (Hillis and Dixon, 1991), are valuable in the phylogenetic separation of closely related species, for the definition of species, subspecies and/or strains (Aktas *et al*., 2007; Jirapattharasate *et al*., 2017).

Liu *et al* (2012) implemented the LAMP (loop-mediated isothermal amplification platform) methodology directed towards specific sequences of the ITS region to detect and distinguish between species in experimentally and naturally infected cattle in China. With a greater sensibility compared to classic PCR for the detection of *B bovis* and *B bigemina*, they highlight the potential of the technique for the detection and differentiation of species, especially in countries where the disease is endemic.

Protein genes associated with roptrias (RAP)

Family of proteins related to the invasión of the parasite and its development in the interior of the host, secreted by the roptrias, bulb-based organelles present in the parasite.

The genetic organization of the RAP-1 locus in most *Babesia* species seems very complex, with at least four different RAP 1A genes in *B bigemina*. The expression of multiple RAP-1A genes containing polymorphic regions and additional genes in the RAP1 family, such as RAP-1B and RAP-1C, result in the production of diverse proteins which can carry out functions that may be identical, different or redundant. In contrast, *B bovis* only has two RAP-1A genes arranged in tandem, so it is assumed that the mechanisms employed for variation in RAP-1A genes and the evolution and organization of the RAP-1 locus may not be the similar in allBabesia species (Suarez *et al*., 1998).

Although some studies have genetically characterized and compared sequences of *B bovis* sbp-4 and *B bigemina* RAP-1 isolates, it is considered that by being highly conserved regions that present homology between different geographical areas, they are useful as specific targets for the detection of the parasite by molecular techniques (Jirapattharasate *et al*., 2017; Roy *et al*., 2018). In *B bovis*, 58 Kda RAP-1 has conserved epitopes and is immunogenic for T and B cells (Mcelwain *et al*, 1987, 1991; Rodriguez *et al*., 1996). It is considered a specific species marker with a greater percentage of identity for *B bovis* of different geographic regions, than for *B bigemina* (Ríos y Ríos, 2011).

In addition to the molecular targets already mentioned, Hosary, 2017 performed the molecular detection of *B bovis*through the amplification of the BV5650 gene, which encodes for a membrane protein, which is different from other proteins of the apical complex, such as RAP-1, which are preserved among different species of Babesia. Its use in the diagnosis of *B bovis*infection can increase the specificity and sensitivity of the PCR (AbouLaila *et al*.,2010).

In general, the study of the species of the genus *Babesia* allows the discovery of genes related to other organisms of the Phylum Apicomplexa, which includes a large number of endoparasites that attack animals. Through comparisons of EST (expressed sequence markers) between *B bovis*, *T gondii* and *P falciparum*, homologous sequences with known genes have been found, increasing their importance for comparative analysis (Sivakumar *et al*, 2014; Pérez de la Rosa *et al.*, 2012).

Reports of prevalence, management practices, vectors and risk conditions

26 of the selected studies analyzed prevalence and all of them were carried out on a local level. With very heterogeneous values, ranging from 0.2% to 62.2% in groups between 76 and

2880 individuals from different categories. Concerning the susceptibility by age groups, there are contrasting results, some studies report that young cattle, between 1 and 2.5 years old, are more affected than cattle older than 2.5 years (Rahman *et al*., 2015), mainly if they are raised under intensive systems; and that crossbreed animals are more affected than animals of Friesian and native breeds (Rizk *et al*., 2017; Jaimes, Triana y Mejía., 2017). In contrast, Roy *et al*., (2018), found that young cattle (less than 2 years old) had lower positive rates of *B bovis* and *B bigemina* compared to older cattle. According to them, this difference could be caused by the greater innate immune response at a young age, a similar conclusion to the one reached by Souza *et al*., (2013), who, considering age, found no statistically significant differences between the seroprevalences of *B bovis*, *B bigemina* and *A marginale*, suggesting that the animals sampled had been infected mainly before reaching one year of age, when they tended to be more resistant to hemoparasites.

The main vectors for *B bovis* are *Rhipicephalus microplus, R. annulatus y R. geigyi*, while *B bigemina* can be transmitted by *R microplus, R annulatus, R decoloratus, R geigyi* and *R evertsi* (OIE, 2008) and *B divergens*is usually transmitted by *Ixodes ricinus*(Guswanto *et al*., 2017). The identification and characterization of protective antigens against ticks has been a growing area of research since the early 1990s; the ability to control infestations through host immunization has been proved using recombinant proteins, however, a limiting step continues to be the identification and characterization of protective antigens (Almazan *et al.,* 2005), because its design has been hindered by extensive polymorphisms in some of the parasite's proteins, particularly those expressed on its surface (Torina *et al.,* 2010).

Ecology, management practices, presence of ticks, age, race and place of origin are potential risk factors for the presence of the parasite. Even animals raised in intensive systems have higher infection rates and there is more diversity of parasites in areas

where there are not tick management practices (Santos *et al*., 2017; Rizk *et al*., 2017). In addition, in many studies co-infections are more common than single *Babesia* infections (Zerihun *et al*., 2017), which could have implications because of the potential interaction between pathogens and clinical symptom patterns (Paolettaa *et al*., 2018; Jirapattharasate *et al*., 2017).

In some countries, like Nigeria, the deficit in veterinary services, causes an indiscriminated use of medications by the producers which leads to problems with antibiotic resistance (Rizk *et al*., 2017). In other countries, like Vietnam, the prevalence of the disease is influenced by the importation of infected livestock that can promote the introduction of new strains, possibly compromising the development of anti *Babesia* immune control strategies, making evaluations prior to importation necessary (Sivakumar *et al*., 2018).

Kubelová *et al* (2012) propose as basic measures to reduce economic losses in livestock production: extensive epidemiological studies using both serology and molecular genetic methods, the monitoring of vector distribution, availability of vaccination programs and traceability of animal transport.

4. Conclusion

The volume of studies found and the systematized information regarding the characteristics of the populations evaluated, the mentioned antecedents, the molecular techniques used and the regions of the genome evaluated in the parasite, show the wide distribution of the disease and the variability of factors that influence its presentation. However, it is evident the need to extend the research in aspects in which the molecular techniques turn out to be very useful tools, such as the particularities in the parasite's genome, the functioning of its genes and its interaction with other parasites and with the host, in order to advance in the development of control and/or eradication strategies.

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Tabla 1. *Blancos moleculares empleados para la detección de Babesia spp. y autores que refieren su aplicación en los estudios seleccionados (2010-2018)*

Parasite	Molecular target	References
Babesia spp.	18sRNA	Ayodele *et al*., 2018; Kubelová *et al.,* 2012; Lempereur *et al*., 2012; Paolettaa *et al.*, 2018; Roy *et al.*, 2018; Byaruhanga *et al*., 2016; Simuunza *et al*., 2011
B bovis	18sRNA	Paolettaa *et al.*, 2018; Roy *et al.*, 2018; Byaruhanga *et al*., 2016; SouzaI *et al*., 2013; Shebish *et al.*, 2012; Simuunza *et al*., 2011; Hosary (2017)
	RAP-1 Rhoptry-associated protein 1a (BbiRAP-1a)	Santos *et al*., 2017; Rizk *et al.*, 2017; Sivakumar *et al.*, 2012; Ybañez *et al*., 2013
	Spherical Body Protein 2 gen (SBP-2)	Herrera *et al*., 2017; Cao *et al*., 2012; Jirapattharasate *et al*., 2017
	Spherical Body Protein 4 gen (SBP-4)	Guswanto *et al*., 2017; Terkawi *et al*., 2012
	Gen BV5650	Hosary (2017)
	Cytochrome B gene (mt-cytB)	Giglioti *et al*., 2017
	Putative gene of babesipsin	Martins *et al*., 2010
	Variant erythrocyte surface antigens (VESA1)	Rahman *et al,* 2015
	Internal transcribed spacers (ITS)	Liu *et al*., 2012
	Merozoite surface antigens (MSA)	Ramos *et al*., 2010
B bigemina	18sRNA	Paolettaa *et al.*, 2018; Roy *et al.*, 2018; Byaruhanga *et al*., 2016; SouzaI *et al*., 2013; Shebish *et al.*, 2012; Simuunza *et al*., 2011; Zerihun *et al*., 2017; Merino *et al.*, 2011
	RAP-1 rhoptry-associated protein 1a (BbiRAP-1a)	Santos *et al*., 2017; Herrera *et al.*, 2017; Jirapattharasate *et al*., 2017; Guswanto *et al.*, 2017; Zhou *et al*., 2016;Terkawi *et al*., 2012; Cao *et al*., 2012
	Apical membrane antigen (AMA-1)	Rizk *et al*., 2017; Musingzi *et al*., 2016; Ybañez *et al*., 2013; Sivakumar *et al*., 2012
	Putative gene of babesipsin	Martins *et al*., 2010
	Internal transcribed spacers (ITS)	Liu *et al*., 2012
B ovata	Apical membrane antigen (AMA-1)	Sivakumar *et al.*, 2012